VPS13A is a multitasking protein at the crossroads between organelle communication and protein homeostasis

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CHAPTER 5

Summarizing discussion and perspectives

SUMMARIZING DISCUSSION

Over 30 years ago, yeast Vps13 was discovered as one of the proteins required for carboxypeptidase Y (CPY) sorting[1–3]. The presence of four human Vps13 orthologues and their associations with the onsets of clinically distinct neurological and developmental disorders[4–7] calls for the demand to mechanistically study each VPS13 protein in multicellular organisms. The challenge has been in defining the localization and functional contributions of each VPS13 family member in molecular pathways.

Vps13 is a very large protein and in fact the fifth largest protein in the yeast proteome[8]. Extraordinarily large proteins often incur technical challenges. It is extremely difficult to clone full-length cDNAs encoding such proteins and equally cumbersome to overexpress them. In this thesis, using *Drosophila* and mammalian cell models, we identify the localization, interaction and an essential role of VPS13A in proteotoxic handling, synaptic structure, organelle contacts and lipid droplet (LD) dynamics.

Localization and membrane association of VPS13A

Mutations in *VPS13A* are associated with Chorea-Acanthocytosis (ChAc)[4]. One of the long standing questions in the ChAc field was to pinpoint the subcellular localization of VPS13A. In this thesis, we provide for the first time, evidence that VPS13A is a peripheral membrane protein, predominantly associated with multiple intracellular organelles. In *Drosophila*, Vps13 is enriched in endosomal fractions (Chapter 2), while in cultured human cells, VPS13A is mainly localized at the ER-mitochondria junctions under normal culturing conditions and is dynamically associated with LDs depending on the cellular lipid content (Chapter 4).

Multiple lines of evidence established that VPS13 family members are membrane bound proteins[3,6,9]. The exact topological arrangement of each protein on intracellular organelles was not demonstrated in these manuscripts. Nonetheless, in several organisms, chemical extraction or enzymatic cleavage methods were used to examine membrane binding properties of VPS13 proteins[3,5,6]. VPS13B and VPS13C are peripheral membrane proteins that are bound to the Golgi and mitochondrial outer membrane respectively[6,9]. Similarly, yeast Vps13 is extracted into the aqueous medium after Triton X-114 treatment while integral membrane proteins do remain in the detergent phase[3]. In chapter 2, we demonstrated that *Drosophila* Vps13 is enriched in membrane fractions and co-immunoprecipitates with Rab7 containing organelles. Furthermore, Vps13 dissociates from membrane compartments upon chemical treatments that also extract the peripheral membrane protein ATP5A[10].

Similar to *Drosophila* VPS13A, human VPS13A also has characteristics of a peripheral membrane protein (chapter 4). VPS13A primarily targets mitochondria through its C-terminal. Although it remains to be investigated whether VPS13A directly binds to mitochondrial outer membrane lipids or is peripherally bound to integral membrane proteins, we do show that VPS13A directly binds to the ER protein VAP-A through the FFAT motif (chapter 4).

VPS13A in Autophagy: structural similarities between VPS13A and ATG2

The coordinated balance between protein translation, assembly and clearance maintains the steadiness of the proteome and governs cellular anatomy and physiology. Failure in proteostasis is a hallmark of many neurodegenerative and age relates disorders[11]. Eukaryotic cells use two major catabolic processes (autophagy and proteosomal degradation) as housekeeping mechanisms to maintain proper balance of their proteome[12]. Macroautophagy (shortly autophagy) is a bulk degradative system where cytosolic contents such as proteins, organelles or microorganisms are engulfed by isolation membranes to form autophagosomes. Autophagosomes subsequently fuse with lysosomes and their contents are digested by hydrolytic enzymes[13]. A number of AuTophaGy related (ATG) proteins have been identified which regulate the autophagic process[14–16]. ATG2 is one of the ATG proteins essential for autophagy and in its absence autophagosome membrane closure is incomplete[17].

The Chorein, ATG-C and APT1 domains of Vps13 show high homology with the N-terminal, C- terminal and APT1 domains of Atg2 respectively[18–20]. Both Vps13 and Atg2 bind to PtdIns3p via their APT domains[20,21]. In cultured cells, both proteins are recruited to LDs upon oleic acid treatment[17,19,22](Chapter 4). In addition, downregulation of either Atg2 or Vps13 in different organisms impairs autophagy, suggesting that both proteins are required for an efficient autophagic flux [15,17,23,24]. It is however not clear whether any of the VPS13A domains are directly involved in autophagic function of mammalian cells. Interestingly, in Chapter 2, we demonstrated that *Drosophila* Vps13 co-fractionates with endosomal markers and specifically immunoisolates with Rab7 positive organelles, suggesting a localization to the degradative compartment[10]. Vps13 also regulates sorting and endosomal recycling of cargo proteins[25]. Indeed, in chapter 2 we revealed that ubiquitinated and autophagic cargo proteins are accumulated in a Vps13 deficient *Drosophila* model, a phenotype that is partly rescued by human VPS13A. Consistently, in ChAc patient cells, autophagic cargo and lysosomal proteins are accumulated and this is associated with delayed mitochondrial clearance[24].

The structure of Vps13 was revealed only recently. Negative stain electron microscopy (EM) combined with single particle analysis showed that Vps13 has a distinctive structure with an extended rod in the middle flanked by a hook at one end and a loop at the opposite end[26]. The flexibility of the middle rod renders the hook and loop to be either arranged in the same or opposite directions. The frequency of these two forms and how the particular conformation is associated with the abundancy of certain lipids and interacting proteins remains to be determined. Interestingly, there is a structural similarity between Vps13 and Atg2. Both VPS13 and ATG2 have a hook and a loop at opposite sides of the protein[26,27] (Figure 1A and B). Insertion of a detectable protein label at different sites of ATG2 followed by single particle analysis revealed that both C- and N-terminal ends are located to the same tip of the folded protein and both ends are located at the opposite site of the loop[27]. It is therefore reasonable to assume that the C-terminal and Chorein domains are tangled together to form the hook end of VPS13A.

Notably, in addition to the previously defined domains, our bioinformatics search using the PRABI coiled-coils prediction programme, revealed that VPS13A contains a coiled-coil region close to the Chorein domain (Figure 1C and D). Coiled-coil domains are key zippers for protein dimerization[28,29]. These observations highlight that dimerization may determine the steric arrangement of VPS13A. It remains to

be shown whether the domains described above are required for localization, stability or proper function of VPS13A in biological systems. Moreover, it would be exciting to investigate whether malfunctioning of specific domains contribute to ChAc.

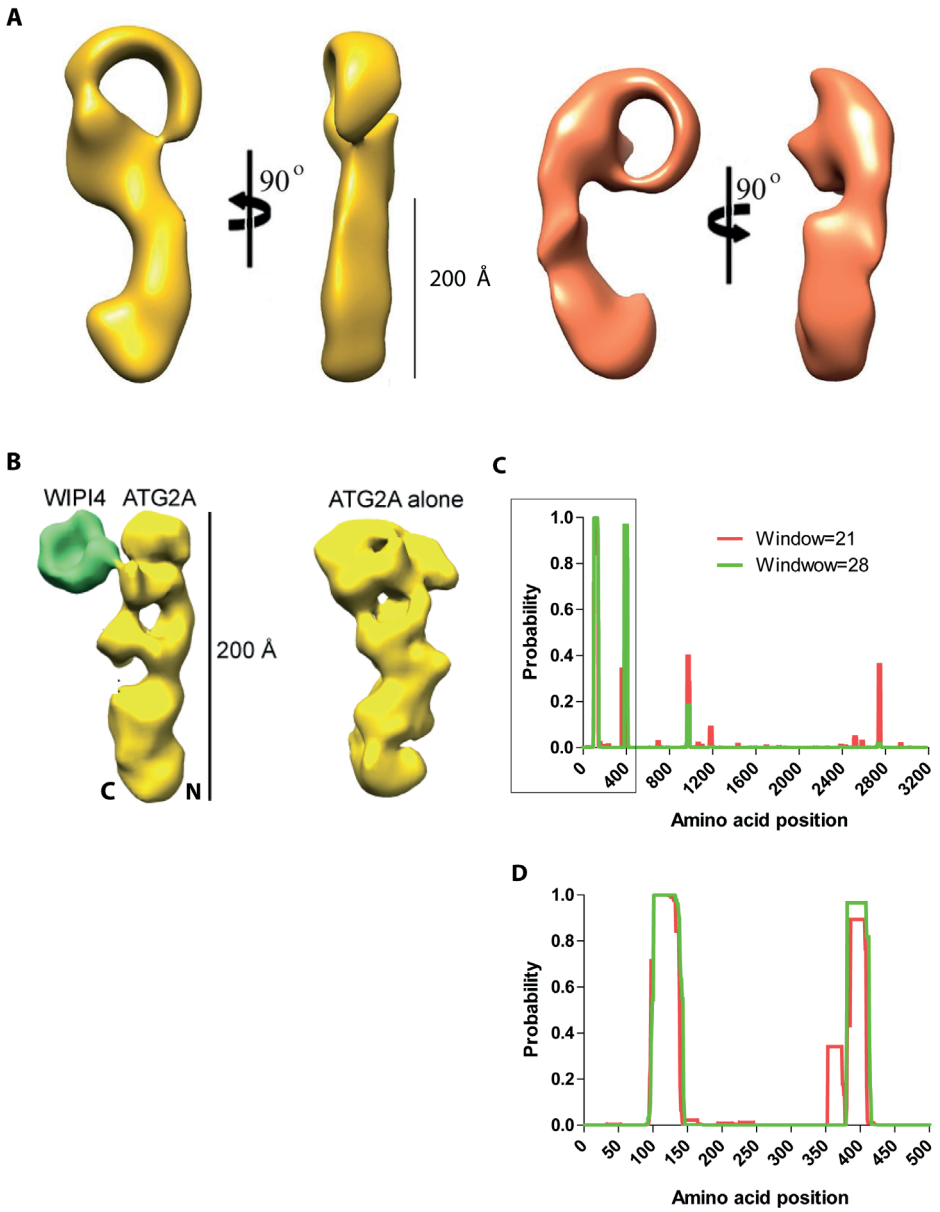

Figure 1. The structure of Vps13.

(**A**) Vps13 typically has a loop on one end and a hook on the opposite.(Adapted from[26]) (**B**) Left panel - ATG2A-WIPI4 complex. Both the N- and C-terminal ends of ATG2A are located at the tip of the rod part. Right panel - ATG2 alone. Adapted from[27] (**C**) Coiled-coil prediction of full length VPS13A. Predictions were done using the PRABI programme. https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_lupas.html. Regions of high probability score are enlarged in **D**.

Actin polymerization defects in ChAc models

Actin is one of the most abundant intracellular proteins in mammalian cells[30]. It is found in either a soluble monomeric form (G-actin) or as polymerized actin (F-actin). Actin cytoskeleton defects have been implicated in many neurodegenerative disorders including ChAc[31–34]. ChAc patients' cells, VPS13A depleted cultured cells and Vps13 mutant yeast cells show an apparent reduction in expression of actin and impaired actin polymerization[32,35,36] which might contribute to the observed abnormal mechanical properties and impaired lamellipodia formation [37].

Dynamic assembly or disassembly of actin regulates many cellular processes including intracellular transport, organelle fission, spine morphogenesis, and synaptic plasticity[31,33,34,38,39]. Interestingly, abnormal outgrowth of NMJ is apparent in Vps13 mutant larvae (Chapter 3). In addition, actin forms immunocomplexes with both mammalian VPS13A and yeast Vps13[20,40]. Along these lines, VPS13A silencing turns down PI3K activity and its downstream targets Rac1 and PAK1; all of which inherently regulate actin polymerization[32].

Actin polymerization is also regulated by phosphoinositides[41–43]. For instance, actin comets are formed in vitro on vesicles that are enriched with both PI(4,5)P2 and Ptdns3p[44]. Increased synthesis of PI(4,5)P2 from Ptdns4p triggers actin comets. Nonetheless, production of PI(4,5)P2 without Ptdns4p consumption induces membrane ruffles indicating that co-regulation of various phosphoinositides determines the fate of actin organization[45]. Vps13 directly binds to a range of phosphoinositides[26]. Furthermore, the amount and distribution of Ptdns4p is significantly impaired in VPS13A depleted PC12 cells, which also show neurite degeneration after NGF stimulation[46], this suggests a link between impaired function of human VPS13A and actin organization defects. It is therefore justified to infer that VPS13 could modulate actin cytoskeleton maintenance possibly by regulating phosphoinositide metabolism.

VPS13A in membrane contact sites

A typical feature of eukaryotic cells is the presence of intracellular compartments known as organelles; each with their own unique lipid and protein composition. Although membrane separation ensures segregation of macromolecules, organelles must communicate in harmony in order for cells to efficiently function as a coherent unit[47–49]. Organelles are not autonomously self-sufficient; their homeostasis is often dependent on proper exchange of information with neighbor-organelles. For proper organelle homeostasis, the right amount of molecules such as proteins and lipids that are distantly synthesized need to be delivered to the target destination at the right time[48].

Transport of lipids and proteins is accomplished through vesicular or non-vesicular transport pathways. Vesicular transport is an ATP dependent process that often involves fusion of organelles. Non-vesicular transport on the contrary is an energy independent, short-range and rapid transport that occurs at specialized bridges (10-30nm) between closely apposed membranes known as membrane contact sites (MCS)[48,50,51].

In the 1950's EM analyses led to the identification MCSs: where people observed very close orientations of highly specialized ER tubules with mitochondria and the plasma membrane (PM)[52,53]. Although the closeness of these membranes was apparently more than what could have happened by chance, it was not clear why membranes of two separate organelles come in contact. It later became obvious that phospholipids can be transferred between mitochondria and microsomes without membrane fusion, suggesting the presence of a separate pathway enabling macromolecule transport other than the vesicular transport system[54,55]. However, it is only recently that the molecular function and architecture of MCSs start to emerge[56].

One of the most remarkable factors that mediate contact sites with ER is the ability of some, if not all, contact site proteins to bind to VAP, a conserved ER resident transmembrane protein[57]. VAP-A/B proteins have been extensively characterized as MCS hubs when the ER establishes membrane contacts with other organelles including endosomes, mitochondria, peroxisomes, the plasma membrane (PM) and the Golgi[28,58–66].

VAP binding proteins contain a specialized stretch of amino acid sequences (FFAT) that enables them to bind to the MSP (major sperm protein) domain of VAP and a second domain that guides their localization to the other organelle[58,60,61,65]. The consensus FFAT motif has a sequence of EFFDAxE, where x can be any amino acid. Amino acid substitutions in the FFAT motif are highly tolerated and proteins that directly bind VAP do not always possess a canonical FFAT sequence. For example, protrudin binds to VAP-A via its FFAT motif (EFKDAIE). Where the natural substitution F>K is tolerated, VAP-A binding of protrudin is inhibited upon D>A substitution[67].

Bioinformatics data mining for proteins harboring FFAT motif pools a number of human proteins including VPS13A and VPS13C[68]. Nonetheless, it is not clear whether all proteins identified are able to bind VAP. Our immunoprecipitation experiments revealed that endogenous VPS13A and VAP-A are pooled in the same complex. Using bacterially expressed VPS13A fragments, it was also shown that VPS13A binds to VAP-A through the FFAT motif (EFFDAPC) and, D>A substitution in the VPS13A-FFAT sequence partially inhibits VAP-A binding. Live tracking of VPS13A with simultaneous visualization of the ER or mitochondria showed that VPS13A is associated with both organelles. Almost every mitochondrion is decorated with VPS13A and is in close association with the ER. Importantly, deleting FFAT from VPS13A restricts its localization only to the mitochondria suggesting that FFAT is an important signal for ER localization of VPS13A (Chapter 4). Altogether, these results uncover the previously unknown localization of VPS13A and its main determinants of organelle recruitment.

Membrane contact sites bridge exchange of molecules such as Ca^{2+} and lipids[69]. The largest cellular store of Ca^{2+} is present in the ER at a millimolar range while cytosolic Ca^{2+} is only in a nanomolar range. This gradient is maintained through coordinated regulation of ER membrane proteins such as Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase SERCA and Inositol-1,4,5-triphosphate Receptors (IP3Rs). The former pumps calcium from cytosol to ER while the latter releases calcium from ER to the cytosol[69,70].

ER Ca^{2+} store depletion activates the interaction of stromal interacting molecule (STIM) and Orai Ca^{2+} entry channels to allow Ca^{2+} influx in to the cell. Experimentally, increased cytosolic Ca^{2+} can be achieved by inhibiting SERCA. Inhibition of SERCA activates Store-Operated Ca^{2+} Entry (SOCE) leading to a massive influx of Ca^{2+} from extracellular medium to the cytosol[71]. The expression of STIM1 and Orai1, and the overall rate of SOCE is diminished in ChAc patient cells[72] suggesting that calcium homeostasis might be mis-regulated in ChAc. Inhibition of SERCA increases the intensity of MCSs between ER and a number of other organelles including PM and mitochondria[73,74]. In cultured cells, inhibition of SERCA increases the interaction between VPS13A and VAP-A suggesting an enhanced formation of VPS13A mediated ER-mitochondria contact sites (Chapter 4). However, the exact role of VPS13A in calcium homeostasis is still unclear.

Synthetic biology screen in yeast uncovered a protein complex bridging ER and mitochondria; hence the name ER-Mitochondrial Encounter Structure (ERMES) was coined. Four core subunits make up ERMES: Mmm1/ Mdm12 /Mdm34 and Mdm10. Mdm10 and Mdm34 are mitochondrial proteins that are in complex with Mmm1 (an integral ER protein) and a soluble Mdm12[75]. Disruption of ERMES does not completely abolish lipid transport suggesting the presence of an independent compensatory mechanism[76]. Identification of vCLAMP (vacuole and mitochondria patch) unveiled the importance of organelle cross-talk as ERMES mutants are synthetically lethal when combined with loss of vCLAMP. Growth defects of ERMES mutants are rescued by the overexpression of vCLAMP. Moreover, the expansion of vCLAMP in ERMES mutants further explains the presence of a co-regulated compensatory mechanism between MCSs to maintain organelle health[77,78].

It is still a subject of debate whether ERMES regulates direct lipid transport between ER and mitochondria[75,79]. In a recent *in vitro* study, ERMES mutants expressing a Vps13 dominant mutant show defective phospholipid transfer from ER to mitochondria but not from mitochondria to ER[80]. On the other hand, Vps13 depleted cells do not have detectable defects in total phospholipid levels or synthesis of lipids that demand ER-mitochondria contact sites[8] suggesting that Vps13 may not directly transfer lipids.

Like Vps13, overexpression of Mdm10 complementing protein (Mcp1) and Mcp2 rescue ERMES mutant-associated growth defects. Both Mcp1 and Mcp2 are mitochondrial proteins and loss of Mcp1 is synthetically lethal when combined with ERMES mutants[81]. An interesting crosstalk between Mcp1 and Vps13 to overcome ERMES mutant growth defects was reported: Mcp1 is mandatory for the Vps13 dominant mutant mediated rescue of ERMES growth defects[8]. Simultaneously, Mcp1 requires Vps13 to function normally [82]. Indeed, Mcp1 and Vps13 interact with each other and overexpression of Mcp1 sequesters Vps13 from vesicles to mitochondria[82].

Loss of ERMES induces an iron deficiency response and leads to iron accumulation[83], a phenotype that is suppressed by the Vps13 dominant mutation. Mcp1 has predicted heme-binding domains and disruption of these domains fail to restore ERMES defects[82]. However future experiments are required to decipher whether VPS13A deficiency causes iron imbalance as a result of MCS malformation and whether such failure contributes to the onset or progression of ChAc. Because the mammalian counterpart of Mcp1 is not known and VPS13A is localized to the mitochondria without overexpression of another protein,

an interesting question remains what determines the localization of human VPS13A to mitochondria. A conceivable hypothesis would be: VPS13A, via one or several of its C-terminal domains, interacts either with mitochondrial membrane protein/s or with outer membrane lipids and thereby exposes its FFAT to the cytosol to make membrane bridges between ER and mitochondria. It however remains to be investigated whether VPS13A and other mammalian VPS13 family proteins recapitulate the reported yeast Vps13 functions at MCSs.

VPS13A in LD homeostasis

In eukaryotes, lipid storage and consumption are in a tightly regulated balance in order to adapt to changes of energy demand and supply[84–86]. Cellular energy is stored in LDs as a form of triacylglycerols and sterol esters[85,87]. The exact mechanism underlying LD biogenesis is unclear. However, there is a growing number of evidence which supports the idea that lipid droplets arise from the ER. The favorable model that depicts LD formation is that neutral lipids accumulate at specific microdomains of the ER between the two leaflets of the bilayer such that a bulge of phospholipid monolayer that contains a neutral lipid core is formed and later bud from the ER[86,88,89].

LDs are not only enriched in lipids. A large number of proteins also target LDs to regulate their synthesis and determine their fate[86,88,90–96]. Protein targeting to LDs is often mediated through the presence of either amphipathic α-helices or hydrophobic hairpins (AH)[92,97,98]. Amphipathic helices serve as membrane curvature sensors[99]. Interestingly, both human VPS13A and yeast Vps13 contain putative amphipathic α-helices[100]. Human VPS13A is predicted to contain a single amphipathic α-helix at position 2959-2982 while yeast Vps13 contains two putative amphipathic α-helices at positions 2889-2911 and 2933-2954 (Figure 2)[100].

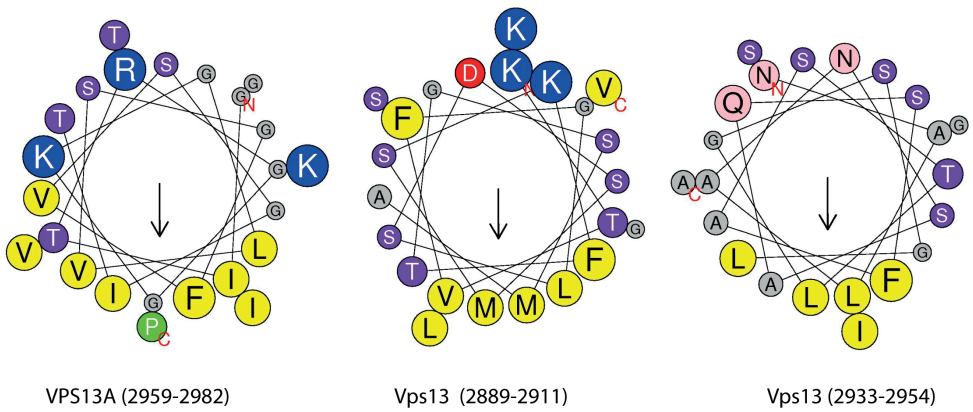

Figure 2. Amphipathic helices of VPS13
Previously predicted[100] amphipathic αhelices of both human VPS13A and yeast Vps13 were analyzed using the heliquest programme. (http://heliquest.ipmc.cnrs.fr/) Hydrophobic amino acids are yellow shaded. Numbers in brackets denote amino acid positions of each stretch

Proteomic analysis of LDs from *Drosophila* embryos identified several proteins including Vps13[94]. Additionally, Vps13 is localized to LDs of seipin mutant cells but not control cells[101]. Seipin is an ER membrane protein that is required for proper formation and maturation of LDs, maintenance of ER-LD contact sites, cargo delivery and modulation of intracellular calcium homeostasis[90,91,95]. Thus, the focal accumulation of Vps13 in LDs of seipin mutants dictates a possible function of Vps13 in LD homeostasis. It is however not clear whether Vps13 is localized in response to a compensatory mechanism of defective LD homeostasis or because of the destabilized ER-LD contact in seipin mutants.

Data presented in this thesis illustrates that human VPS13A is recruited to LDs when cellular lipid is surplus. Fatty acid supplementation and subsequent localization of VPS13A to LDS is not accompanied with an elevated VPS13A translation but with a reduction of VPS13A signal from mitochondria (Chapter 4). We also show that VPS13A negatively influences LD motility. LDs are slow when they are associated with VPS13A and move faster directionally when they are dissociated from VPS13A. Moreover, VPS13A deficient flies and human cells show impaired LD homeostasis. Together, these results reflect a conserved function of VPS13A in LD dynamics perhaps by establishing MCSs between LDs and neighboring organelles.

MCSs govern organelle mobility: such that endosomes and peroxisomes are mobile upon loss of tethering complex[60,61,102]. We therefore predict that loss of MCSs in VPS13A depleted cells might abrogate lipolysis and/or FA transfer to other organelles and consequently account for an increase in LD size number. Of note, increased LD size creates a surface tension that is unfavorable for lipases[103,104].

Upon nutrient deprivation, cells undergo lipolysis to liberate fatty acids (FAs) from lipid droplets. The released FAs are immediately taken up by the mitochondria for ATP production through the process of β-oxidation[105]. Under such conditions, mitochondria reside in close proximity with LDs likely for the efficient transfer of FAs[105,106]. In addition, nutrient starvation promotes mitochondrial elongation and fragmented mitochondria are evident in cells that do lack mitochondrial fusion proteins such as mfn1 and Opa1[63,105]. Such mutants do have increased number and size of LDs likely because they reroute free fatty acids back to LDs for esterification and storage[105]. It is therefore tempting to investigate the role of VPS13A in mitochondrial respiration and morphological organization.

Impaired LD homeostasis in neurodegenerative diseases

In the nervous system, lipids play vital roles, such as myelination, synaptic integrity and global metabolism[107–109]. Mutations of lipid binding proteins and changes in brain lipid profiles have been implicated in neurodegeneration[110–113]. However until recently, the role of LDs in the nervous system has not been appreciated. LD accumulation has been documented in mouse models as well as in lymphoblasts and brain slices of Huntington's disease patients[114]. The role of LDs in the *Drosophila* brain have been comprehensively investigated[115–118] and, in chapter 4, we demonstrated that LDs accumulate in glia cells of Vps13 deficient flies.

There appears to be a cross talk between neuronal and glial cells to properly handle lipotoxic insults. For example, glial LDs sequester toxic lipid species and thereby safeguard neuronal precursors from oxidative

attacks[118]. Glia specific down regulation of mitochondrial complex I leads to lipid droplet accumulation and neurodegeneration[117]. In mutants with neuronal mitochondrial dysfunction, LDs accumulate in glia cells non-cell-autonomously. Neuronal mitochondrial dysfunction liberates reactive oxygen species (ROS) that triggers the activation of sterol regulatory element binding protein (SREBP) and c-Jun-N-terminal-kinase (JNK) pathways. Activation of these pathways enhances excessive formation of glial LDs and subsequently promotes neurodegeneration[115]. The exact mechanism of such non-cell autonomous regulation is not completely understood, but a recent study has been illuminating. Inter-cellular shuttling of lactate from glia to neurons fuels neuronal lipid formation during ROS production. Hereto, specific fatty acid transporter channel lipids to glial cells[116].

CONCLUDING REMARDKS AND PERSPECTIVES

Data presented in this thesis not only defines a previously unidentified localization and interaction of VPS13A but also highlights a conserved importance of VPS13A to regulate LD dynamics in both mammalian cell lines and *Drosophila* ChAc model. We believe that the function of VPS13A is not limited to what is reported in this thesis. However, considering the key evidences and available tools, we believe that the molecular pathways governing ChAc are within reach. Because LD accumulation and impaired protein homeostasis were documented in our ChAc models, it would be interesting in the future to link VPS13A's role in autophagy and LD homeostasis. The question of how impaired LD dynamics contributes to ChAc remains to be solved. Therefore, future study involving the function of VPS13A in lipid biosynthesis and transport, membrane contact integrity and ER-LD-mitochondria interplay would give us insights to better understand the pathophysiological processes of ChAc and to further identify druggable targets.

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